The Pharma Innovation Journal 2019; 8(9): 244-248

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(9): 244-248

www.thepharmajournal.com
Received: 16-07-2019
Accepted: 18-08-2019

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Effect of volume of washing fluid on bacterial load in preputial wash, fresh and post-thaw semen in crossbred bulls

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Abstract
Bacteria present in the semen cause harmful effect on the spermatozoa through toxin production and generated metabolic end products that challenges the future fertility. It is now well known that preputial cavity is one of the main sources of contamination in ejaculated semen. Despite of the fact, there is no standard methodology and recommendations to eliminate such contaminations from prepuce prior to semen collection. Considering the above facts, the present study was designed to examine the effect of different volume of flushing fluid to reduce this preputial contamination in semen. Total preputial capacity (100%) was determined in 11 crossbred bulls by in-housed designed PDC (Preputial Douching and Cleaning) device and subsequently two different volumes i.e. 150 mL (50%) and 215 mL (70%) were calculated based on the full capacity keeping conventional 100 mL as control. The preputial bacterial load increased (P<0.05) significantly with the increase of washing fluid volume from 100 mL to 302 mL, Following washing, at day 1, the mean bacterial count in fresh semen significantly decreased (P<0.05) with the increase of washing fluid volume except 302 mL volume in which the count characteristically increased (P<0.05). At day 5 and 10, the same trend was also observed as in day 1, showing a decreased (P<0.05) mean bacterial count with the increase of washing fluid volume except 302 mL in which the count was increased (P<0.05) significantly. In post-thaw semen also, the bacterial count at day 1, 5 and 10, decreased (P<0.05) significantly in a similar fashion as in fresh semen following preputial wash.

Keywords: Crossbred bull, bacterial load, preputial wash, fresh, post-thaw semen

1. Introduction
Microbial contamination of semen remains the subject of research investigations since many years. However, the particular area has drawn massive research attention after the realization by OIE regarding the transmission of diseases through semen and consequently many recommendation/guidelines are emerged for all semen producing stations throughout the globe. Among the microbes, bacterial contamination plays an important role for the future fertility of semen once inseminated in female animals (Prince *et al*., 1949) [20]; (Boryczko *et al*., 1985) [4]; (Griveau *et al*., 1995) [11]; (Diemer *et al*., 1996) [6]. Usually, healthy bulls are either free or with very low number of bacteria in the reproductive tract (Gilman, 1921) [10]. Despite the facts, no semen ejaculate obtained so far are reported to be completely free from bacteria even after exploring all the precautions taken prior to collection (Hatziolos, 1937) [14]; (Meena *et al*., 2015) [17].

Bacterial flora of semen arises from many sources like unhygienic surrounding, repeated entry of penis in to artificial vagina**,** improper handling of semen**,** age of bulls, order of ejaculate and inflammatory condition of reproductive system (Gunsalus *et al*., 1941) [12]; (Foote and Sffalisburry, 1948) [7]; (Amquist *et al*., 1949) [2]; (Jone *et al*., 1964) [15]. Of all the external sources, preputial cavity is considered to be the main source of contamination in ejaculated semen (Prince *et al*., 1949) [20]; (Palec and Kazda, 1961) [18]; (Frank and O'Berry, 1962) [8]; (Boryczko *et al*., 1985) [4]. It is thus recommended to regularly flush the preputial cavity just before semen collection to reduce the bacterial count in the semen (Gunsalus *et al*., 1941) [12]; (Zemjanis, 1962) [24]; (Galloway 1964) [9]; (Hashimoto, 1966-1967) [13]; (Reddy *et al*., 1971) [21].

Available reports on the allied line suggest that preputial washing is a basic prerequisite to reduce the level of bacterial contamination/load in semen. Further, it is reported that the bacterial load of semen may vary among the age, species, season, breed and types of prepuce

of the donor bulls (Reddy *et al*., 1971) [21]; (Kher and Dholakia, 1987) [16]; (Sannat *et al*., 2015) [22]. Sporadic reports are also available regarding the volume of washing fluid (100 mL or 200 mL) to be used for preputial washing without any specific recommendation for determining the volume of washing fluid. Variable bacterial counts in washing fluid as well in the semen were also appeared in various reports. Furthermore, to investigate the effects of elimination of bacterial load in semen, majority used 100 mL NSS led to inconsistent results (Kher and Dholakia, 1987) [16]; (Bindra *et al*., 1994) [3]; (Meena *et al*., 2015) [17]. However, to the best of our knowledge, reports pertaining to the effect of volume of preputial washing fluid on bacterial load of fresh and frozen semen are not available in existing literature. In the present study we examined the effect of different volume of washing medium, determined based on the preputial capacity, on elimination of bacterial load in washing fluid and fresh as well as frozen semen in crossbred bulls.

2. Materials and methods

2.1 Climate and Location

The work was carried out in the Germ-Plasm Centre (GPC) of Animal Reproduction Division, Indian Veterinary Research Institute, Izatnagar, India which is located at 28°10' North latitude and 78''23' east latitude at an altitude of 172 meter above the mean sea level. The temperature of the region varies between 8^0C (winter months) and 40^0C (summer months). The average rainfall is 1087.9mm and rainy seasons starts in June and extend up to September with humid and warm conditions.

2.2 Experimental animals

A total of 11 healthy crossbred bulls were selected for determining the preputial capacity. Later on three bulls were used for each method of washing with a specific volume of fluid. All the bulls were healthy and sexually active.

2.3 Nutrition and management

The experimental animals were maintained under identical feeding and managerial conditions during the entire course of the study. The bulls were supplied with concentrate ration (5 kg), green fodder (25 kg) and roughages (5 kg) once a day with ad libitum fresh drinking water.

2.4 Experimental designs

2.4.1 Determination of Preputial capacity

The optimum preputial capacity was determined based on the maximum volume of the fluid that the organ withstands. Preputial hairs were trimmed properly and the area around preputial orifice was cleaned before the start of experiment. Preputial capacity was measured by using Preputial douching and cleaning device (PDC device) developed by this laboratory (Prasad *et al*., 2016) [19] for the purpose of preputial washing of bulls (Fig.1). The sterilized normal saline solution was filled through the water inlet (2.5lits) valve of this device and closed tightly, and then air was pumped into the air inlet (30 to 35 psi) valve. The nozzle of this device was then inserted through preputial orifice to a depth of an inch into the preputial cavity. Preputial orifice was kept tightly closed by holding the skin fold tight, to prevent the back flow of the fluid. The NSS was passed to full capacity of preputial cavity while keeping the orifice closed. The nozzle was withdrawn while keeping the preputial orifice tightly closed. After releasing the grip over the preputial orifice the fluid was drained out and collected into a autoclavable polythene bag. The volume of drained out fluid was measured using graduated measuring cylinder and the actual volume was noted. This technique was repeated three times to each bull to find out the average preputial capacity in crossbred bulls.

2.4.2 Procedure of Preputial washing

Sterile normal saline solution was prepared as per standard method. A total of three healthy breeding bulls were used to carry out the experiment. Washing of prepuce was done by two methods *viz*. Traditional or syringe method and PDC device method (Fig.2). In Syringe method (control group), preputial washing was performed with 100 mL NSS using hypodermic syringe (100 mL capacity) fitted with sterilized plastic sheath (6 inches length) which is a traditional method commonly used at most of the semen collection centres. In PDC device method, three different volumes of NSS was used for the preputial washing *i.e.* 50%, 70% and 100% of preputial capacity (Table 1). After inserting the desired volume of NSS in the preputial cavity, penile tract of each bull was massaged thrice from downward to upward direction while keeping the orifice closed and then wash fluid was drained out and collected into an autoclavable polythene bag. Since, same bulls were used in each group hence; an interval of 10 days was given between each experimental method to nullify the effect of preceding method.

Fig 1: Preputial douching and cleaning device

i) Sampling of preputial wash

Preputial washings of all the bulls were performed at '0' day and subsequently wash samples were collected for the bacterial load estimation. A 10 mL volume of drained fluid was immediately used for bacterial load estimation.

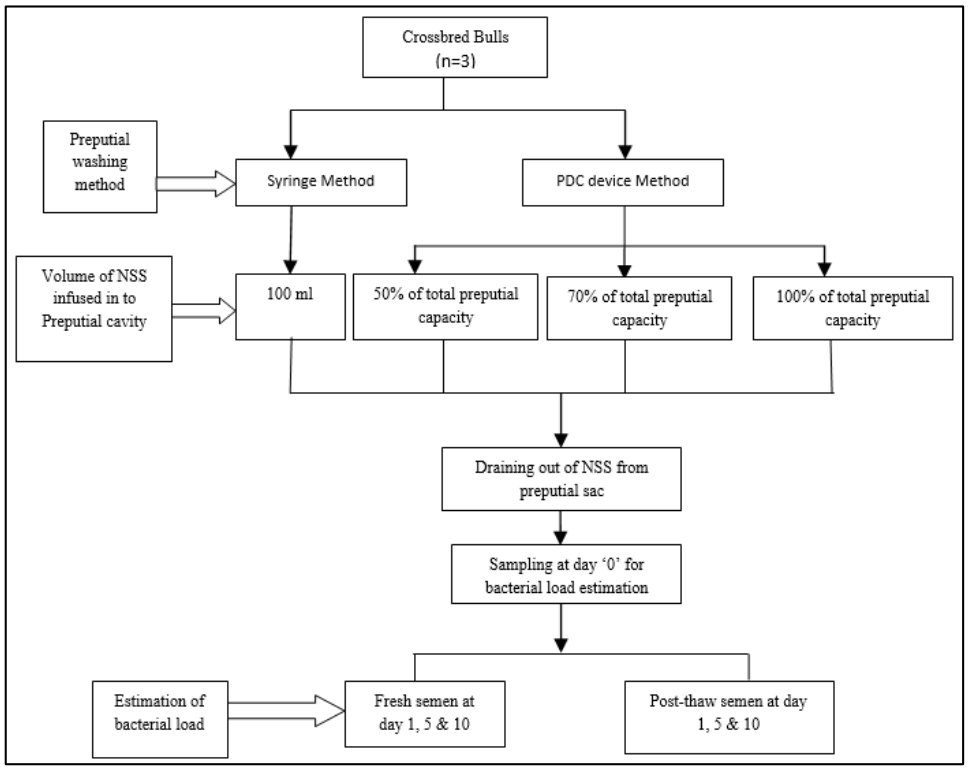

Fig 2: Experimental designs of preputial washing and semen sampling

ii) Semen sampling

Fresh semen from all the groups were collected on day 1, 5 and 10 and frozen as per standard protocol and bacterial load was assessed both at fresh and post-thaw stage. Bacterial load was counted in preputial washing fluid, fresh and post-thaw semen using standard plate count.

iii) Standard Plate count

The samples processed for standard plate count were collected under strict aseptic condition. Fresh semen samples processed for bacterial counting were used within half an hour of collection while post-thaw semen samples after 24h of freezing and the standard plate count were done by spread plate technique in duplicate plates. Tenfold serial dilution from 1: 10 to 1: 1000 were made for all preputial washing samples for standardization of SPC. Subsequently 1:10 to 1:100 dilutions were used for bacterial count from preputial washing samples and 1: 10 was used for bacterial count from fresh and post-thaw semen samples. The normal physiological saline solution was used as routinely diluents for all samples. The inoculated nutrient agar plates were allowed to dry for 10 minute before incubation at 37^0C for 24 h. The bacterial count (cfu/mL) of each sample was counted by multiplying the dilution factor with number of colony in plate.

2.5 Statistical analysis

One way ANOVA was used to analyse the differences among different volume of washing fluid groups to study the effect of different volume of preputial washing fluid on mean bacterial load of prepuce, fresh semen and post-thaw semen. Results were presented as mean±SE. Tukey test was used to compare mean between different volumes groups of washing fluid. The analysis was done by using (SAS 9.3) [23] software.

3. Results

The mean bacterial load obtained in preputial wash, fresh and post-thaw semen is presented in Table1. In the present study, the bacterial load increased significantly (P<0.05) with the increase of washing fluid volume from 100 mL to 302 mL. However, the difference in the mean count remained similar between the 150 mL and 215 mL groups (Table 1). Further, when we increased the volume up to 215 mL, the bacterial load was still in lower in number. However, the bacterial count of this study showed a linear increase with the increase of the volume of washing fluid from 100 mL to 302 mL.

Following washing, at day 1, the mean bacterial count in fresh semen significantly (P<0.05) decreased with the increase of washing fluid volume except 302 mL volume in which the count characteristically increased (P<0.05). Similar trends in bacterial count were also observed at day 5 and day 10 in fresh semen following washing. At day 5 and 10, the mean bacterial count significantly (P<0.05) decreased with the increase of washing fluid volume except 302 mL in which the count was (P<0.05) significantly increased (Table 1). Further, when we increased the volume of washing fluid from 100 mL to 215 mL, the count was characteristically reduced from 3.63 x 10^3 to 1.56 x 10^3 cfu/mL. At day 5 and 10 the bacterial count decreases in same fashion as at day 1.

In post-thaw semen, at day 1 also showed a similar trend as observed in fresh semen following washing. It revealed a decreasing (P<0.05) trend in the bacterial count with the increase of washing fluid volume except 302 mL volume in which count was increased significantly (P<0.05) in the same fashion as in fresh semen. Similar trends were also recorded at day 5 and day 10 following washing in post-thaw semen. At day 5 and 10, the mean bacterial count was significantly (P<0.05) decreased with the increase of washing fluid volume except 302 mL in which the count was increased (P<0.05) unexpectedly (Table 1).

When the volume of fluid was further increased up to the total capacity *i.e.* 302 mL, the bacterial count was significantly increased in same trend as in fresh semen at day 1, day 5 and day 10.

4. Discussion

The present study demonstrated that increasing the washing volume of fluid increases the elimination of prepucial bacterial population thereby decreases the bacterial count in the fresh as well as post-thaw semen following preputial washing.

A regular flush of the preputial cavity is a basic prerequisite to reduce the level of bacterial load in semen (Gunsalus *et al*., 1941) [12]; (Zemjanis, 1962) [24]; (Reddy *et al*., 1971) [21]. Washing hastens the elimination of bacteria thereby reduces the bacterial count in the semen collected in Artificial vagina (AV). Generally, 100 or 200 mL NSS or PBS are reported to be used for washing of prepuce prior to semen collection that resulted with a variable bacterial count (59 to 672.0 x10^3cfu / mL) in preputial wash (Reddy *et al*., 1971) [21]; **(**Kher and Dholakia, 1987) [16]; (Bindra *et al*., 1994) [3]; (Ahmed *et al*., 2001) [1]; (Meena *et al*., 2015) [17]. In the present study, the bacterial load increased significantly (P<0.05) with the increase of washing fluid volume from 100 mL to 302 mL. However, the difference in the mean count remained similar between the 150 mL and 215 mL groups (Table 1). The bacterial count obtained after washing with 100 mL NSS of this study was higher than that reported in other studies (Kher and Dholakia, 1987) [16]; (Bindra *et al*., 1994) [3]. Further, when we increased the volume up to 215 mL, the bacterial load was still in lower in number compared to a previous study (Reddy *et al*., 1971) [21]. However, the bacterial count of this study showed a linear increase with the increase of the volume of washing fluid from 100 mL to 302 mL. This indicates that an optimisation of the volume perhaps increased the total area intended for cleaning resulting into more number of bacterial colonies in the preputial wash.

Table 1: Effect of volume of washing fluid on bacterial load (mean±SE) in preputial wash, fresh and post- thaw semen in crossbred bulls

Volume of fluid	Bacterial Load (x10^3cfu/ml)						
	Preputial wash	Fresh semen			Post-thaw semen		
	Day '0'	Day 1	Day 5	Day 10	Day 1	Day 5	Day 10
100ml (Control)	151.00±7.00^c	3.63±0.13^a	4.25±0.31^a	4.65±0.02^a	2.43±0.17^a	2.93±0.28^a	3.3±0.30^a
150ml	178.66±4.97^b	2.26±0.06^b	2.96±0.08^b	3.5±0.28^b	1.5±0.05^b	1.76±0.08^b	2.26±0.12^b
215ml	185.33±5.78^b	1.56±0.12^c	2.06±0.08^c	2.53±0.14^c	0.56±0.08^c	0.83±0.08^c	1.16±0.08^c
302ml	232.00±4.93^a	3.93±0.08^a	4.63±0.13^a	5.1±0.11^a	2.6±0.15^a	3.2±0.26^a	3.7±0.25^a

Means with different superscripts in a column differs significantly (p<0.05)

The bacterial count obtained in the fresh semen at day 1 following washing of this study was comparable to that reported in other crossbred bull (Kher and Dholakia, 1987) [16], which was lower than that (57.0 x 10^3 cfu/mL) reported previously using 200 mL NSS (Reddy *et al*., 1971) [21]. The significantly lower number of bacterial count in fresh semen following washing with 215 mL of sterile NSS by the present method indicates a better approach for elimination of bacterial load. On the other hand when the volume of fluid was further increased up to the total capacity *i.e.* 302 mL, the bacterial count was significantly increased, which was a reverse in the trend. It is possible that the volume of fluid corresponding to the total preputial capacity caused wound because of pressure injury to tissue. This may lead to the invasion of exogenous bacteria, because exposure of subcutaneous tissue following loss of skin integrity (*i.e.*, a wound) provides a moist, warm and nutritious environment conducive to microbial colonization and proliferation (Bowler *et al*., 2001) [5] resulting in to the increased bacterial count. The injury of the cavity was further substantiated by the appearance of more number of epithelial cells in the washing fluids and also in the fresh semen (unpublished observation).

The bacterial load recorded in post-thaw semen at day 1 following washing of prepuce by 100mL NSS was not conformity with the previous reports (0.23x10^3 to 1.31x10^3 cfu/ mL) by (Bindra *et al*., 1994) [3]; (Meena *et al*., 2015) [17]. This variation in result might be due to species differences. Further, when we increased the volume of washing fluid from 100 mL to 215 mL, the count was strikingly reduced from 2.43 x 10^3 to 0.56 x 10^3 cfu/ mL, which was lower than that (1.31 x 10^3 cfu/mL) reported earlier practicing 100mL NSS washing (Bindra *et al*., 1994) [3] while the result was also not conformity with the finding (0.23x10^3 cfu/mL) by (Meena *et al*., (2015) [17]. The reason of this difference may be species specificity. At day 5 and 10 of post-thaw semen the bacterial count decreases in the same fashion as at day 1 except 302 mL. The decreasing of bacteria count significantly in post-thaw semen as in fresh semen following washing with 215 mL of sterile NSS by the present method indicates a better approach for elimination of bacterial load.

On the other hand when the volume of fluid was further increased up to the total capacity *i.e.* 302 mL, the bacterial count was significantly increased in same trend as in fresh semen at day 1, day 5 and day 10. The region of this increase in bacterial count was suspected to be same as it was observed in fresh semen.

Strengths and limitations of the study: An apparent limitation of the study was the absence of a PDC 100 mL group, which limits the possibility of a direct comparison with the traditional syringe method using 100 mL flush volume. Owing to this limitation, the results of this study should be interpreted with caution, considering a potential confounding effect of the PDC device on the results

5. Conclusion

The results of this study suggest that increasing the volume of preputial washing fluid from the existing 100 mL volume to 215 mL may increase the elimination of preputial bacterial population and decrease the bacterial load in both fresh and post-thaw semen. However, a further increase of the volume according to the total prepucial capacity may not be much beneficial requires future confirmation.

6. Acknowledgements

The authors are thankful to Director, Indian Veterinary Research Institute, Izatnagar, Bareilly (UP) Pin code -243122 for providing facilities and fund during thesis research work of first author. We are also thankful to all the staffs of GPC for their help during restraining of animals.

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